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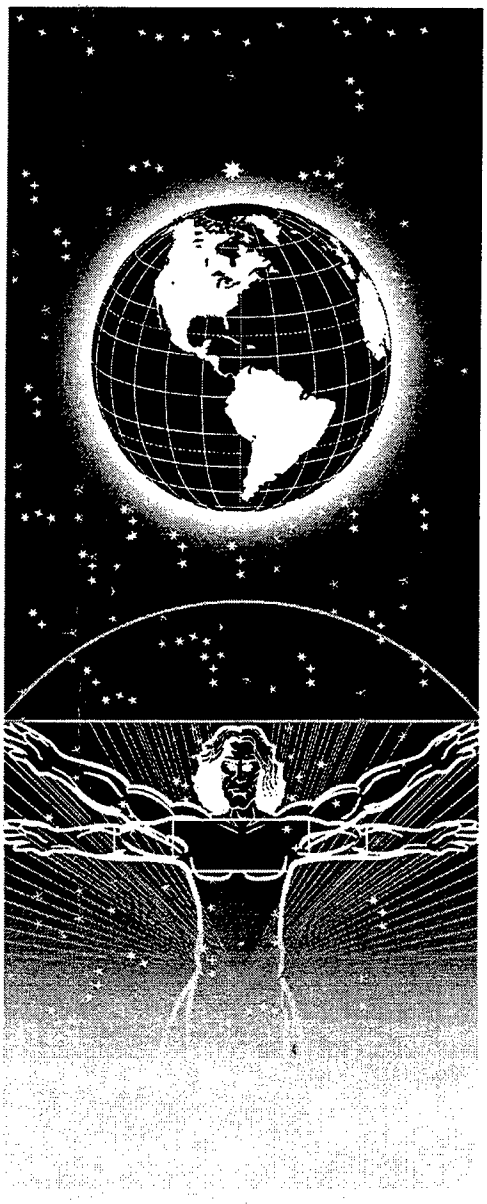

UNITED STATES AIR FORCE
ARMSTRONG LABORATORY

QUANTITATIVE ASSESSMENT OF PEROXISOME PROLIFERATION IN B6C3F1 MOUSE LIVER AFTER SUBCHRONIC EXPOSURE TO TRICHLOROETHYLENE BY GAVAGE

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July 1997

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TECHNICAL REVIEW AND APPROVAL

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The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

TERRY A. CHILDRESS, Lt Col, USAF, BSC
Director, Toxicology Division
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Trichloroethylene (TCE), an industrial solvent and common groundwater contaminant, is hepatocarcinogenic in mice when given at high doses. One potential mechanism for TCE-induced liver tumors is the formation of excessive reactive oxygen species during lipid peroxidation. As reported in previous studies by analysis of protein levels, enzyme activity or transmission electron microscopy (TEM) examination of liver tissue, TCE exposure results in peroxisome proliferation. In this study, groups of mice were orally administered water, corn oil, or TCE (400, 800, 1200 mg/kg) in corn oil in equal volumes for 8 weeks (once/day, 5 days/week). We present data showing significant induction of mRNA levels for cytochrome P450 4A1 in high dose groups (3-30 times control) and glyceraldehyde-3-phosphate dehydrogenase (1.4-3.4 times control), as determined by Northern analysis throughout the study. The induction of these genes is closely associated with peroxisome proliferation. In addition, gamma-glutamylcysteine synthetase mRNA displayed elevations in high dose groups (1.3 to 3.6 times control). The implication of peroxisome proliferation was verified by TEM examination and quantitation of peroxisome numbers. The evidence of peroxisome proliferation-associated gene induction throughout the 56-day exposures suggests that peroxisome proliferation could play a continuing role in the biological effects of a longer-term TCE exposure.

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PREFACE

This one of a series of technical reports describing the results of experimental laboratory programs conducted at OL AL HSC/OET. This document serves as an interim report on the subchronic toxicity of trichloroethylene (TCE) in mouse liver. The pervasive use of trichloroethylene in the past has resulted in its being a clean-up priority for the future. This research was conducted to better understand the mechanisms of TCE toxicity in the mouse, in order to better establish the potential hazards for humans. The results of this study provide some of this necessary insight by describing the response of biomarkers of TCE exposure, namely peroxisome proliferation and associated gene expression. The research described in this report began in July 1994 and was completed in November 1996 under U.S. Air Force Contract Nos. F33615-90-C-0532 (ManTech Environmental, Inc.), F41624-96-C-9010 (ManTech/Geo-Centers Joint Venture) and Department of the Navy Contract No. N00014-95-D-0048 (Geo-Centers, Inc.). LtCol Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

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I. INTRODUCTION

Trichloroethylene (TCE) is a solvent and degreasing agent used widely by industry and on Department of Defense installations. It is a common groundwater contaminant and has been shown to be hepatocarcinogenic in rodents (NCI, 1976; NTP, 1988, 1990). Human risk estimates for cancer have been largely based on mouse liver cancer data. To establish that the human risk guidelines are reasonable, it is necessary to understand the carcinogenic mechanism of TCE in mice and to determine whether similar risks are associated with human exposure (Goldsworthy and Popp, 1987; Drinkwater, *et al.*, 1989; Ashby, *et al.*, 1994; Xu, 1995).

The toxicity of TCE is associated with its metabolism (Moslen, *et al.*, 1977; Goeptar, *et al.*, 1995; Templin, *et al.*, 1993). Mouse liver tumors have resulted from exposures to three major TCE metabolites: chloral hydrate (CH), trichloroacetic acid (TCA), and dichloroacetic acid (DCA) (Herren-Freund, *et al.*, 1987; Daniel, *et al.*, 1992; DeAngelo, *et al.*, 1991). Only CH has been found to be genotoxic (reviewed in ECETOC, 1994). The others are considered non-genotoxic carcinogens.

Mechanisms of non-genotoxic carcinogenesis have been proposed that are associated with activation of steroid hormone-like receptors (Green, 1992, Melnick, *et al.,* 1996). Chemical agents that induce peroxisome proliferation interact with similar receptors (peroxisome proliferator associated receptors or PPARs). The roles of peroxisome proliferation in the mechanism of carcinogenesis are not fully understood. Prolonged exposure of rodents to xenobiotics that cause peroxisomal proliferation results in several responses in the liver (hepatomegaly, hepatocellular hyperplasia, increased peroxisomal compartment) and eventually hepatocellular carcinoma. These pleotropic effects are thought to be mediated by PPARs (Aldridge, *et al.,* 1995). These receptors function by binding response elements upstream of genes and activating their transcription. Activation of proto-oncogenes by PPARs in this manner could serve to promote tumorigenesis.

An alternative hypothesis for the mechanism of TCE-induced hepatocarcinogenesis is based on oxidative stress within the liver cells. PPARs, by binding response elements, induce formation of peroxisomes along with several genes encoding enzymes of the peroxisomal β-oxidation pathway, namely acyl-CoA oxidase and acyl-CoA thiolase (Aldridge, *et al.*, 1995). These enzymes are thought to result in generation of excess hydrogen peroxide (H_2O_2), a potential source of free oxygen radicals (Gulati, *et al.,* 1993). These radicals can bind DNA or signal transduction mediating molecules and potentially modulate gene transcription and the cell cycle. These events can ultimately lead to the promotion of tumorigenesis (Burdon, 1995; Byczkowski and Channel, 1996; Green, 1992; Melnick, *et al.,* 1996; Janssen, *et al.,* 1993; Van der Vliet and Blast, 1992). The role of free radical generation by oxidative stress in tumor promotion has been well reviewed (Kozumbo, *et al.*, 1985; Thrush and Kensler, 1991; Janssen, *et al.*, 1993). Studies have demonstrated that reactive oxygen species may alter the regulation of cell growth by disrupting signal transduction molecules and receptors without directly attacking nuclear DNA (reviewed in Van Der Vliet and Bast, 1992; Burdon, 1995; Byczkowski and Channel, 1996).

One event that can play a role in the induction of an oxidative stress environment in rodents is an increase in peroxisome proliferation (Arnaiz, *et al.,* 1995). Peroxisome proliferation has been shown to occur in mice treated with either TCE or its metabolites (Elcombe, 1985; Chang, *et al.*, 1992; and Austin, *et al.*, 1995). In mice, chronic TCA treatment resulted in sustained peroxisomal proliferation, whereas the effects were transitory for DCA (Bull, *et al.*, 1993). This transitory DCA effect on peroxisome proliferation, coupled with the fact that chronic exposure of mice to DCA causes liver tumors, suggests that other factors besides peroxisome proliferation may play a significant role in DCA-promoted hepatocarcinogenesis. The complexity of peroxisome proliferation's role in hepatocarcinogenesis is certainly not diminished by the mixed evidence for TCE's (and metabolites TCA and DCA) role in inducing peroxisome proliferation in the rat (Parnel, 1985; Elcombe, 1985 and Odum, *et al.,* 1988).

Other findings implicated TCA and DCA treatment in the induction of oxidative stress within the liver tissue, independent of peroxisome proliferation (Parrish, *et al.*,

1996 and Nelson, *et al.*, 1989). Both TCA and DCA increased the formation of thiobarbituric acid-reactive substances (TBARS) in a dose-responsive manner, suggesting that each chloroacetate was capable of yielding a radical species that could initiate lipid peroxidation (Larson and Bull, 1992). Recently, CH and TCA were shown to generate free radicals and induce lipid peroxidation in male B6C3F1 mouse liver microsomes (Ni, *et al.*, 1996). This laboratory has shown that carbon-centered free radicals are produced in B6C3F1 liver slices exposed to the parent compound TCE (Steel-Goodwin, *et al.*, in press). Furthermore, we have observed the formation of TBARS, DNA adducts and periods of cellular proliferation in mouse liver after TCE gavage (unpublished data). Thus the oxidative and peroxisome proliferation events may both be contributing factors to the hepatocarcinogenic effect of TCE in the B6C3F1 mouse.

A number of genes can be monitored as biomarkers of oxidative stress or peroxisomal proliferation events. Increases in cytochrome P-450 IVA1 (CYP4A1) levels in animals treated with known peroxisome proliferators can be dramatic. CYP4A1 is transcriptionally regulated (Webb, *et al.*, 1996) and is coordinated with the transcription of β-oxidation enzymes, acyl-CoA oxidase and thiolase. This resolution precedes the peroxisomal response and is therefore reported as an important marker of peroxisome proliferation (Aldridge, *et al.*, 1995). Previous studies confirm the induction of CYP4A1 messenger RNA (mRNA) by TCE and other haloalkanes (Wang, *et al.*, 1996). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a key metabolic enzyme in glycolysis, has been shown to increase in association with exposure to known peroxisome proliferators (Rumsby, *et al.*, 1994). Variation in G3PDH mRNA levels is a rarely observed phenomenon. Additionally, the induction of gamma-glutamylcysteine synthetase (GCS) mRNA is associated with oxidative stress in the cell (Woods, *et al.*, 1992). The GCS enzyme catalyzes the first step of glutathione (GSH) synthesis and is responsive to feedback inhibition by GSH (Moslen, *et al.*, 1977; Seelig, 1984). The relative effects of TCE exposure on both G3PDH and GCS mRNA have not been previously reported.

The purpose of this study was to assess early cellular responses to a 56-day subchronic exposure to TCE by corn oil gavage. Potential biomarkers of response (mRNA analysis) were used to investigate the potential relationships between peroxisomal proliferation (identified by electron microscopy) and potential oxidative stress as early events during TCE treatment.

II. MATERIALS AND METHODS

Animal Exposures and Sampling

Male B6C3F1 mice (12 weeks old; 25-30 g) were obtained from the Charles River Breeding Laboratories. Mice were housed 5 mice per plastic cage, provided with hardwood chip bedding and maintained on a 12-hour light/dark cycle at a constant temperature of 22±1°C and humidity of 35-50%. The mice were provided with *ad libitum* water and Purina Formulab #5008.

Trichloroethylene (99.5+% without antioxidant additives) was obtained from Aldrich (Milwaukee, WI, Lot No. MF 01428EF). Gavage solutions were prepared fresh each week in corn oil (Mazola™, Best Foods, Somerset, NJ). All gavage solutions were stored at 4°C. Animals were dosed total volume of 0.5ml (0, 400, 800, or 1200 mg TCE/kg) five days a week for 8 weeks. One dose group was given only water, no oil or TCE. Each dose group contained seven mice for each time point. (Table 1).

TABLE 1. STUDY DESIGN

Treatment	No. of mice	Time points	Total no. of mice
Water control	7	11	77
Corn oil control	7	11	77
TCE 400 mg/kg/day	7	11	77
TCE 800 mg/kg/day	7	11	77
TCE 1200 mg/kg/day	7	11	77

Liver samples were obtained on days 2, 3, 6, 10, 14, 21, 28, 35, 42, 49, and 56 on dose, beginning 3-4 hours after morning dose. Thirty minutes prior to beginning the euthanization by rapid CO_2 asphyxiation, an intraperitoneal injection of N-t-butyl-a-phenylnitrone (PBN), 200 mg/kg, was administered to trap radical species for electron paramagnetic resonance analysis. Preliminary work in this laboratory had determined this dose of PBN to be nontoxic and not to interfere with subsequent analyses. Livers were quickly removed, and washed in ice-cold Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO BRL, Grand Island, NY) (pH 7.4). Separate samples for RNA analysis and TEM were taken from each liver. Samples for RNA, TBARS, and electron paramagnetic resonance analysis were immediately snap frozen in liquid nitrogen and stored at -140°C. Samples for TEM were glutaraldehyde-fixed.

Transmission Electron Microscopy (TEM)

Slices of liver for electron microscopy were diced into 1mm cubes and fixed in 2% glutaraldehyde on 0.1M sodium cacodylate buffer (pH7.4) for at least 24 hrs. Fixed samples were rinsed 4 times in cacodylate buffer, post-fixed in 1% OsO_4 in 0.1M sodium cacodylate buffer (pH7.4) for 2 hours and dehydrated in a graded ethanol series. After two changes in propylene oxide, tissues were embedded in Poly/Bed 812 (Polysciences, Warrington, PA) according to Glauert (1974). Thin sections (60-90nm) were cut on a MT7000 (RMC, Tucson, AZ) ultramicrotome, counterstained with 3% uranyl acetate followed by Reynold's lead citrate and viewed in a JEOL 1200 EXII TEM (JEOL, Peabody, MA) at 60kV. Micrographs of representative centrilobular hepatocytes immediately adjacent to the central vein were taken at 10,000x. Peroxisomes in these micrographs were manually demarcated and then quantified using computer-based morphometric analysis (Quantimet 570c, Leica, Inc., Deerfield, IL).

RNA Analysis

Total RNA was extracted from liver tissue using a commercial reagent (RNA Stat 60) and dissolved in specially prepared formamide (Formazol), both from Tel-Test "B", Inc. (Friendswood, TX). Extracted RNA was stored at -140°C until Northern analysis.

RNA samples were electrophoresed (40ug/lane) on 1% formaldehyde gels and transferred to positively charged nylon membranes (MAGNA-NT, Micron Separations, Inc., Westboro, MA). Membranes were then UV-crosslinked and baked at 80°C for 1hr.

The CYP4A1 cDNA probe was made by PCR amplification of a 1342bp fragment from a plasmid clone of rat CYP4A1 obtained from Dr. James P. Hardwick (North-eastern Ohio Universities College of Medicine, Roostown, OH). Primers were designed using OLIGO 4.0 software (NBI, Plymouth, MN) and synthesized on an ABI 392 DNA synthesizer (Perkin-Elmer Corporation, Norwalk, CT). The primer sequences were: 5'-CATGAGCGTCTCTGCACTGA-3' and 5'-AGGAATGAGTGGCTGTGTCG-3'. The g-GCS heavy chain (GCS-H) cDNA probe was a 390bp restriction fragment from a plasmid clone of rat GCS-H from Dr. Terrance Kavanagh (Univ. of Washington, Seattle, WA). The cDNAs were labeled with [32]P-dCTP by random primer method. The GAPDH RNA probe was prepared from a commercial template (AMBION, Austin, TX) by *in vitro* transcription and labeled with [32]P-UTP (NEN, Boston, MA). Hybridizations of cDNAs were performed at 42°C and at 68°C for the GAPDH riboprobe. After overnight hybridization, membranes were exposed to X-ray film at -70°C for 1 to 5 days. Band intensities on developed films were quantified by laser scanning densitometry (Bio-Rad Instruments Model 670 Densitometer with Molecular Analyst Software, Version 1.3, Bio-Rad, Laboratories., Hercules, CA). Equivalent loading among gel lanes of RNA was determined by hybridization with an 18S rRNA probe (AMBION, Austin, TX).

Statistical Analysis

Dose response data were analyzed by one-way ANOVA using BMDP (BMDP Statistical Software, Inc., Los Angeles, CA). Pairwise comparisons for all data were made between dose groups by paired t-test. Treatment differences were considered significant for $p<0.05$. For data presented as percent of control, standard error is reported as a standard error of the ratio between the high dose and oil control group means. Results of the analysis of mRNA for dose response are presented as relative densitometry units. Analysis of mRNAs from multiple days were generally performed over many individual autoradiographs. To control for variation in exposure and development between films, gene expression levels were reported as a percent of control for the samples from that time point.

For analysis of peroxisomal area to total cytoplasmic area, a two factorial analysis of variance was used, applying Levene's criteria to test the assumption of equality of variances.

III. RESULTS

Peroxisome Detection by TEM

Only samples from the 1200 mg TCE/kg, oil and water control groups were analyzed. Days 6, 10, and 14 were chosen based on previous analysis of these tissues, which showed that cellular proliferation was highest during the period of 6-14 days on dose (data not shown). As summarized in Table 2, there exists a treatment and time effect for percent peroxisomal area, a treatment-only effect for number of peroxisomes, and no effect for peroxisomal size. Hepatocytes examined from corn oil control mice were no different than those from water control mice for all the peroxisomal parameters. TCE at 1200 mg TCE/kg significantly ($p<0.05$) increased the percent peroxisomal area of total cytoplasmic area compared to vehicle (corn oil) controls at days 6, 10, and 14. Moreover, total peroxisomal area per unit area of cytoplasm (% peroxisomal area) peaked at exposure day 10. For days 10 and 14, TCE treatment at 1200 mg TCE/kg resulted in a significantly ($p<0.05$) higher number of peroxisomes per unit area of cytoplasm than either corn oil or water treatment alone. For day 6, TCE treatment at 1200 mg TCE/kg differed significantly ($p<0.05$) only from corn oil in the number of peroxisomes per unit area of cytoplasm.

Northern Analysis

Analysis of dose-response relationships was performed on samples from days 2, 3, and 28 for CYP4A1 mRNA to investigate the effects of the shorter (2-3 day) exposures versus those at an intermediate point in the study. Figure 1 shows relative band intensities of northern blots as determined by densitometry. No significant differences in mRNA expression levels were detected between oil control and water control group samples. CYP4A1 mRNA levels were significantly greater ($p<0.05$) for the 400, 800, and 1200 mg/kg groups compared to the water and oil controls. No significant differences were detected between the 400 and 800 mg/kg dose groups. CYP4A1

mRNA for the 1200 mg/kg group had a significant increase over 800 mg/kg for all three days examined. Samples from days 2 and 28 for the 1200 mg TCE/kg group were significantly different compared to the 400 mg/kg group. Based on the small differences between water and oil control groups and the maximal response exhibited by the highest dose groups shown in Fig. 1, samples from only the oil and 1200mg TCE/kg dose groups were chosen to be studied further.

TABLE 2

PEROXISOMES[a]

Exposure day	Group	Percent peroxisomal area[b]	Number of peroxisomes[c]	Peroxisomal size[d]
6	1200 mg/kg TCE	2.87±0.30	48.3±6.9	6.05±0.43
	Corn oil	1.16±0.15[e]	18.7±2.0[e]	6.19±0.20
	Water	1.41±0.22	17.1±1.8[e]	8.69±2.30
10	1200 mg/kg TCE	6.39±0.60[f]	64.2±5.2	10.04±1.14
	Corn oil	1.38±0.21[e]	20.6±1.9[e]	06.65±0.73
	Water	1.16±0.39[e]	14.6±4.1[e]	07.76±1.90
14	1200 mg/kg TCE	4.69±0.22[f,g]	48.2±3.4	9.84±0.93
	Corn oil	0.77±0.08[e]	9.5±0.9[e]	8.10±0.68
	Water	0.88±0.03[e]	10.2±1.9[e]	9.50±2.04

[a] Data are given as mean ± SE; n = 3 per group.
[b] Calculated using 100 x peroxisomal area/total cytoplasmic area examined.
[c] Number of peroxisomes per 10 millimicrons^2 of cytoplasm.
[d] Calculated using total peroxisomal area/total number of peroxisomes; units are 10^4 nanometers^2.
[e] Significantly ($p<0.05$) different than 1200 mg/kg TCE.
[f] Significantly ($p<0.05$) different than day 6.
[g] Significantly ($p<0.05$) different than day 10.

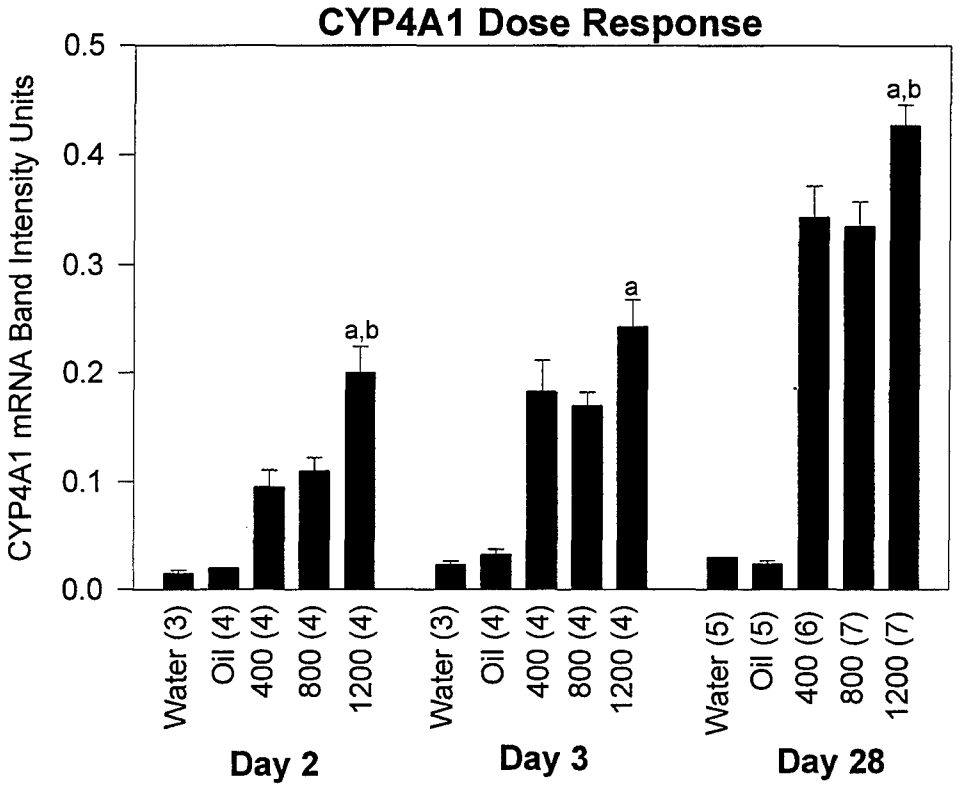

FIGURE 1.
Relative expression (mean ± se) of CYP4A1 mRNA plotted as hybridization band intensities. Results are grouped by day of sacrifice (2,3,28) and by dose (water, oil, 400/800/1200 mg TCE/kg) with sample size (n) indicated below each group. All TCE doses were significantly different from water or oil groups ($p<0.001$). Other significant results are: (a, different from 400mg TCE/kg at $p<0.05$) and (b, different from 800mg TCE/kg at $p<0.05$).

The levels of CYP4A1 gene expression as percent of oil control are shown for the 1200 mg/kg dose group in Figure 2. CYP4A1 mRNA levels for the 1200mg/kg dose group were elevated throughout the exposure period, with the greatest average elevation of 3050% (at $p<<0.001$) seen on day 56. Lesser elevations of 400% and 1050% over control occurred on days 2 and 6, respectively. The other days all showed significant increases ($P<<0.001$) of about 1450% (day 3), 2400% (day 10), 1500% (day 14), 1450% (day 28), and 3050% (day 56).

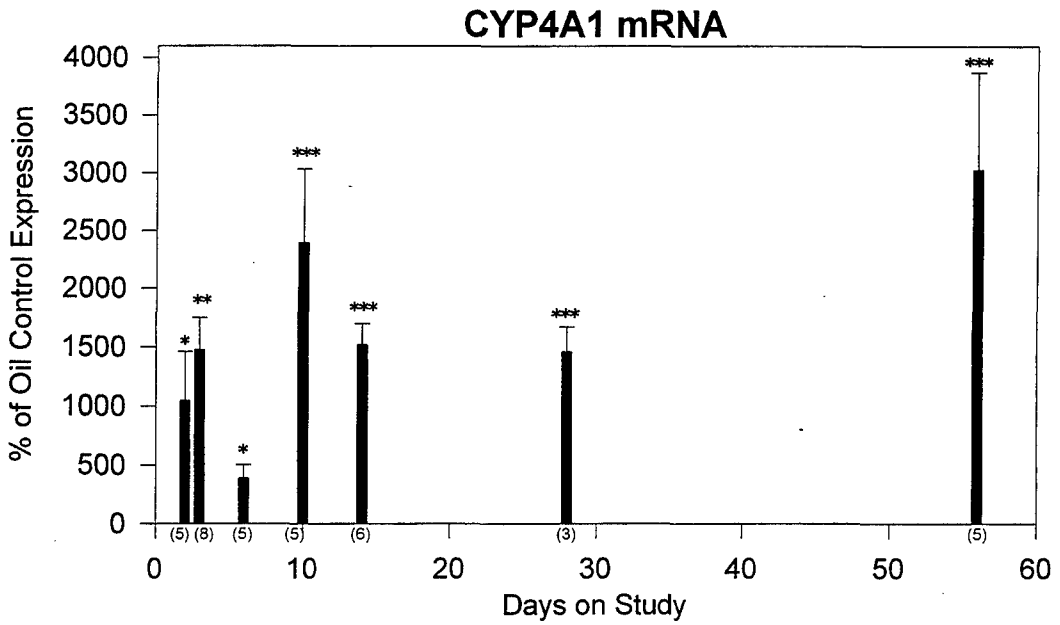

FIGURE 2.
Expression of CYP4A1 mRNA for the 1200mg TCE/kg group as a percent (mean ± se) of that for the oil control group on that day. Sample size (n) is shown below each bar. Significant increases versus control are identified: (*,p<0.05), (**,p<0.01), and (***,p<0.001)

Throughout the study, GCS mRNA levels (Fig. 3) were seen to be elevated (up to 350%) in the 1200mg/kg TCE dose group samples compared to the oil control groups. Significant differences were observed for days 14, 28, 49, 56. Levels were about 150% ($p<0.01$), 200% ($p<0.05$), 350% ($p<0.05$), and 200% ($p<0.01$) over controls, respectively.

G3PDH mRNA levels in the 1200 mg/kg TCE dose group samples were elevated (150-350%) throughout the study (Fig. 4). Levels of G3PDH mRNA were significantly greater than those of oil controls on days 3 (200%, $p<0.01$), 10 (150%, $p<0.01$), 14 (150%, $p<0.05$), 48 (350%, $p<0.001$) and 56(150%, $p<0.01$).

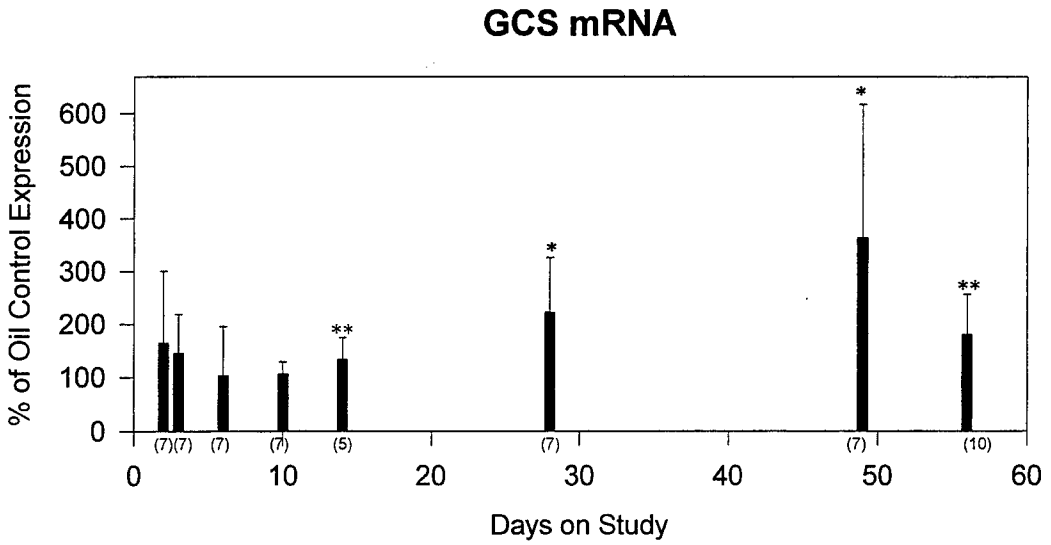

FIGURE 3.
Expression of GCS mRNA for the 1200mg TCE/kg group as a percent (mean ± se) of that for the oil control group on that day. Sample size (n) is shown below each bar. Significant increases versus control are identified: (*,$p<0.05$), (**,$p<0.01$) and (***,$p<0.001$).

IV. DISCUSSION

The results of the molecular and ultrastructural assessments of the liver tissues from the TCE-exposed mice suggest that peroxisome proliferation is occurring, which likely involves induction of oxidative stress. The electron microscopic analysis of peroxisomal proliferation are consistent with those previously reported (Elcombe, 1985). The confirmation of peroxisome proliferation in these samples allows us to analyze the induction of the various genes of interest in light of this event. The levels of peroxisome proliferation as identified by TEM parallels the expression of CYP4A1 mRNA on days 6, 10, and 14.

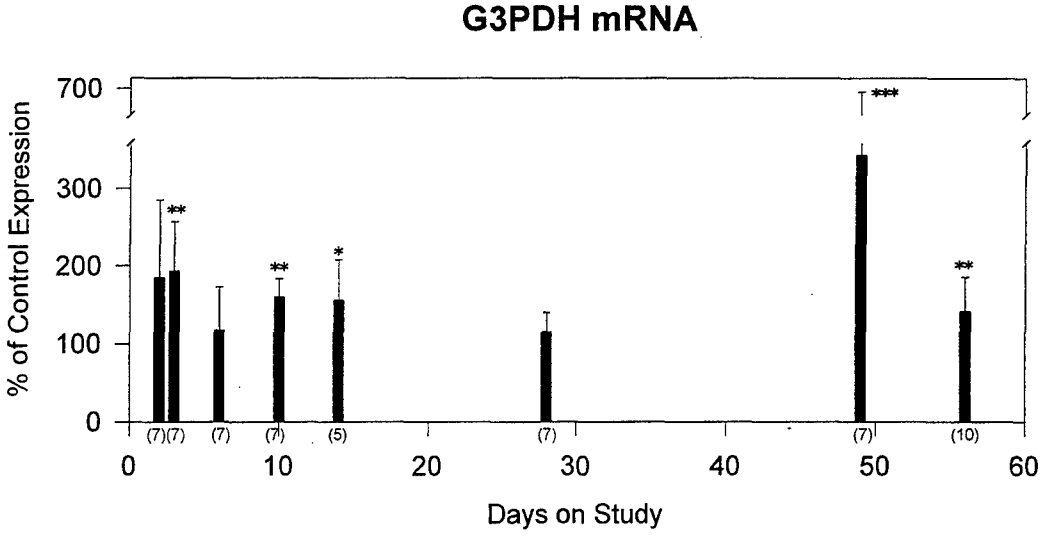

FIGURE 4.
Expression of G3PDH mRNA for the 1200mg TCE/kg group as a percent (mean ± se) of that for the oil control group on that day. Sample size (n) is shown below each bar. Significant increases versus control are identified: (*,$p<0.05$), (**,$p<0.01$).

Expression of CYP4A1 is regulated at the level of transcription (Webb, *et al.*, 1996). Thus it is useful to monitor CYP4A1 mRNA levels as a measure of response to, in lieu of determining protein levels or enzymatic activity. In addition, the same mRNAs can be assayed for many other gene targets without re-purification. Increases of up to 2000% for CYP4A1 mRNA levels have been reported in association with a number of different peroxisome proliferators, such as clofibric acid (Bars, *et al.*, 1993; Prough, *et al.*, 1994). The present study shows the rapid response to TCE exposure of CYP4A1 mRNA after just 2 days of exposure to the highest dose. The response is sustained through day 56. This sustained increase together with the dose response trend (Fig. 1) indicates that CYP4A1 is a good biomarker for TCE exposure. Similar results have been reported for CYP4A1 mRNA after exposure to clofibric acid (Bars, *et al.*, 1993). The present study also indicated that CYP4A1 expression declined from day 3 to day 6 , then increased from days 6 to 10. These data suggest that the consistent elevation of this gene's expression may be dependent on the consecutive daily exposure, since the sacrifice on day 6 was performed after a 2-day (weekend) suspension of the dosing.

Moreover, this suggests the correlation between the timing of TCE exposure and CYP4A1 expression.

The pattern of GCS expression, in relation to TCE exposure, (Fig. 3) suggests a physiological adaptation in the cell, by which GSH biosynthesis is upregulated. As the rate limiting enzyme in glutathione synthesis, GCS plays an important role in regulating glutathione production. Glutathione is instrumental in protecting the cell from oxidative damage. In another study (Woods, *et al.*, 1992), rats were administered methyl mercury hydroxide. After three weeks, the animals exhibited a marked elevation in GCS mRNA. The authors suggest that this was an adaptive function by increasing GSH biosynthesis in response to chemically-induced oxidative stress. This suggestion is supported by reports of increased GCS mRNA levels, which resulted from an oxidative environment induced by elevated amounts of intracellular H_2O_2 (Pan, 1993). We have previously showed that exposure of mice to TCE results in lipid peroxidation, and free radical production (Steel-Goodwin, *et al.*, 1996; Channel, *et al.*, 1997), which may contribute to oxidative stress. The peroxisome proliferation, as determined by TEM and CYP4A1 mRNA analysis, and elevated GCS mRNA levels are consistent with an oxidative stress environment. It appears that the oxidative challenge must be at a level to substantiate an increase in GCS mRNA expression, since others have shown that GCS mRNA induction after oxidative challenge does not always ensue (Davis, *et al.*, 1993). The ability of the cell to cope with the oxidative challenge may be accounted for by GCS mRNA stabilization to support increased GCS protein synthesis, as suggested by Woods, *et al.* (1992) without increasing mRNA levels. Nonetheless, in our case, GCS mRNA levels do increase, indicating a response to a persistent oxidative challenge throughout the dosing regimen.

This oxidative event can also be implicated in the induction of G3PDH mRNA expression. An effect on G3PDH due to experimental manipulations is not generally expected, thus its conventional use as a reference standard in northern analysis experiments (see Wong, *et al.,* 1992, for example). The significantly elevated levels of G3PDH in this study (Fig. 4) are comparable to results reported for exposure to four other known peroxisome proliferating agents (Rumsby, *et al.*, 1994) . One hypothesis to

explain this increase involves the effects of free radicals on the G3PDH enzyme. This interaction could cause a loss of active G3PDH enzyme, possibly related to membrane damage. However, we did not evaluate the effects on other membrane-associated enzymes. A similar decrease in enzymatic activity was previously described resulting from free radicals and excessive H_2O_2 (Nath, *et al.*, 1994; Mallozzi, *et al.* (1995). Furthermore, G3PDH mRNA levels were seen to increase in hepatocellular adenomas and carcinomas induced by diethylnitrosamine, a known inducer of hydroxyl radicals (Hacker, *et al.*, 1991). Alternatively, induction of G3PDH may result from peroxisome proliferators' effects on hepatic intermediate metabolism by changing the cell's utilization of the glycolytic pathway, often increasing b-oxidation, thereby changing the level of the cell's dependence on G3PDH enzyme function (Rumsby, *et al.*, 1994, Hacker, *et al.,* 1992). A specific peroxisome proliferator-responsive element for the G3PDH gene not been identified. Taken together, the literature and our data suggest that peroxisome proliferation and oxidative stress events display correlative increases in G3PDH mRNA levels. These increases in G3PDH mRNA levels are indicative of similar events causing peroxisome proliferation and the responses of CYP4A1 and GCS mRNA.

V. CONCLUSIONS

The ultrastructural assessment confirms peroxisomal proliferation in the liver of B6C3F1 mice following exposure to TCE by oral gavage. Increased expression of CYP4A1, GCS, and G3PDH mRNAs are effects of TCE exposure. The correlation of the CYP4A1 mRNA levels with the TEM analysis further substantiates previous work showing that elevation of CYP4A1 gene expression is an effective early marker of peroxisome proliferation and can be closely linked to exposures to high doses of TCE. The peroxisome proliferation event continues through 56 days, based on the CYP4A1 data. The induction of both GCS and G3PDH mRNAs is consistent with an oxidative stress environment. This has been further corroborated by the detection of increased lipid peroxidation in these samples (Channel, *et al.,* 1997). The oxidative challenge and peroxisome proliferation events are factors which may have a primary effect in the hepatocarcinogenesis process in B6C3F1 mice exposed to TCE by oral gavage.

It has been shown that xenobiotics have not been implicated in causing peroxisome proliferation in humans (Huber, *et al.,* 1996, Richert, *et al.,* 1996). Thus, it is important to define the specific contribution of peroxisome proliferation to rodent hepatocarcinogenesis versus other mechanisms, which may also result from oxidative stress (Parrish, *et al.,* 1996, Nelson, *et al.,* 1989). Further evaluation of the suitability of the rodent model for human TCE exposure risk for liver cancer could include assessment of other TCE exposure mRNA biomarkers, such as GCS and G3PDH.

VI. REFERENCES

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